

II. RESPONSE TO OFFICIAL ACTION OF SEPTEMBER 29, 2004

A. Status of the Claims

Claims 31-53 were pending in the case at the time of the Official Action, dated September 29, 2004. All claims stand rejected. Claims 31-42 stand rejected under 35 U.S.C. § 112, first paragraph. Claims 31-53 were also rejected for obviousness-type double patenting over claims 1-29 of U.S. Patent No. 6,709,664 and claims 1-29 of U.S. Patent No. 6,461,619. Finally, Claims 31-53 stand rejected under 35 U.S.C. § 103(a) over Tatton *et al.* (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash *et al.*, or over Patent No. 5,783,606 to Tatton in view of Quash and Tatton *et al.*

B. Rejections Based on 35 U.S.C. § 112, First Paragraph, are Overcome

Claims 31-42 stand rejected under 35 U.S.C. § 112, first paragraph, because according to the Action, “the specification, while being enabling for reducing photodamage to skin cells, does not reasonably provide enablement for preventing photodamage.”

While Applicant respectfully disagrees that the present specification is not enabled for preventing photodamage, in an effort to facilitate prosecution Applicant has amended claims 31-42 so that they are directed to reducing photodamage to skin cells, but not preventing photodamage to skin cells. Since the Action has acknowledged that the claims are enabled for reducing photodamage to skin cells, Applicant respectfully requests that the Examiner withdraw this rejection. Applicant takes this action merely to allow the claims to progress to issuance, and reserves the right to pursue claims drawn to any canceled subject matter in this or a related patent application.

C. Rejections Based on Double Patenting are Overcome

Claims 31-53 stand rejected for obviousness-type double patenting. The Action cites claims 1-29 of U.S. Patent No. 6,709,664 and claims 1-29 of U.S. Patent No. 6,461,619 as the bases for the double patenting rejections. Applicant has attached hereto a terminal disclaimer that overcomes the rejection over claims 1-29 of U.S. Patent No. 6,709,664 and claims 1-29 of U.S. Patent No. 6,461,619. Somerset Pharmaceuticals, Inc. owns U.S. Patent Nos. 6,709,664 and 6,461,619. Accordingly, Applicant respectfully requests withdrawal of this rejection for obviousness-type double patenting.

D. Rejections Based on 35 U.S.C. § 103(a) Are Overcome

Claims 31-53 stand rejected under 35 U.S.C. § 103(a) over Tatton *et al.* (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash *et al.*, or over Patent No. 5,783,606 to Tatton in view of Quash and Tatton *et al.* The Action states that “Tatton et al teach deprenyl (same as selegiline) for reducing neuronal apoptosis caused by oxidative free radical damage and the reduction is mediated by a principal metabolite of deprenyl, desmethyldeprenyl (same as desmethylselegiline).” The Action also states that Patent No. 5,783,606 to Tatton “teaches deprenyl and desmethyldeprenyl compounds for the treatment of glaucoma.” Finally, the Action states that Quash “teaches modulation of apoptosis (induce or suppress) as a mechanism to prevent or provide treatment for photoinduced or chronological aging of skin and other related skin conditions.” The Action combines these references and asserts that it would have been obvious “for one of ordinary skill in the art at the time of the instant invention to use the anti-apoptotic compounds (deprenyl and desmethyldeprenyl) of Tatton et al (Neurology) for inhibiting or suppressing apoptosis in several dermal or epidermal conditions such as aging...”

Applicant respectfully traverses this rejection because a skilled person would not assume that any anti-apoptotic compound can “prevent aging and its signs such as wrinkles.” For example, the well-known antioxidant beta-carotene has not been convincingly demonstrated in most clinical studies to protect against skin photodamage (See Biesalski *et al.*, Arch. Biochem. Biophys. 389:1, 1-6 (2001), attached as Exhibit A). Beta-carotene has been shown to have anti-apoptotic properties in hepatic and brain cell lines (Bagchi *et al.*, Gen. Pharmac. 30(5):771-76 (1998), attached hereto as Exhibit B; See also Ortmann *et al.*, Radiat. Res. 161(1):48-55 (2004) (“When given prior to irradiation, beta-carotene and vitamin E reduced the amount of radiation-induced apoptosis significantly...”). Bagchi *et al.* found that beta-carotene was able to reduce TPA-induced hepatic and brain DNA fragmentation by 11% (p. 774). Since “[f]ragmentation of nuclear DNA is a biochemical hallmark of apoptosis,” this finding demonstrates that beta-carotene has anti-apoptotic properties (p. 774). Since the anti-apoptotic compound beta-carotene has not been shown to protect against skin photodamage, the statement by Quash relied on in the Action is refuted, and the method of treating or promoting the healing of photodamaged skin with the anti-apoptotic compounds selegiline and/or desmethylselegiline would not be obvious to one of skill in the art.

In addition, the broad statement by Quash that any species capable of modulating apoptosis can also prevent and/or combat the appearance of aging is not supported by the specification of Quash. Quash is more narrowly drawn to methods of modulating apoptosis with methional, malondialdehyde, or factors influencing the intracellular concentrations of methional or malondialdehyde. Therefore, one of skill in the art would consider Quash’s broad statement unsupported and unreliable.

Finally, to establish a *prima facie* case of obviousness, “there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of skill in the art, to modify the reference or combine reference teachings.” MPEP § 706.02(j). If cited references can be properly combined, *O’Farrell* provides a three prong test for determining whether the combined references establish a *prima facie* case of obviousness. *O’Farrell* held that for a combination of references to render a claimed invention obvious the references must provide to one of skill in the art:

- (1) a detailed enabling methodology for practicing the claimed invention;
- (2) a suggestion for modifying the prior art to practice the claimed invention; and
- (3) evidence suggesting that the invention would be successful.

In re O’Farrell, 7 U.S.P.Q.2d 1673 (Fed. Cir. 1988).

There is no teaching or suggestion in any of the cited references, either alone or in combination, of the subject matter of the present disclosure. As admitted in the Action, Tatton and Tatton et al. do not teach treating a subject for photodamage skin or inhibiting photodamage; nor do these references suggest or motivate one of skill in the art that selegiline or desmethylselegiline can be used to treat photodamaged skin. In addition, Quash does not teach selegiline or desmethylselegiline, nor does it support its suggestion that any species capable of modulating apoptosis can also treat photodamage. Thus, a skilled person would not assume that all anti-apoptotic compounds can treat photodamaged skin.

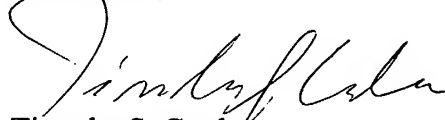
Applicant finds no teaching in the references cited by the Examiner or any other references of record that suggest or teach that selegiline and/or desmethylselegiline would be useful for the treatment of photodamaged skin as disclosed in the present specification. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejections based upon Tatton, Tatton et al., and Quash.

III. CONCLUSION

In light of the foregoing remarks, Applicant respectfully submits that all claims are in condition for allowance, and solicit an early indication to that effect. Should Examiner Channavajjala have any questions regarding this response, please contact the attorney of record, Margaret Sampson, at (512) 542-8569.

Please date stamp and return the enclosed postcard evidencing receipt of these materials.

Respectfully submitted,



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MINIREVIEW

UV Light, Beta-carotene and Human Skin—Beneficial and Potentially Harmful Effects

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Solar radiation is one of the most important environmental stress agents for human skin, causing sunburn, premature skin aging, and skin cancer. Beta-carotene is discussed to protect against photooxidative stress and thus prevent skin damage. Though beta-carotene has been successfully used against photosensitivity in patients with erythropoietic protoporphyria, its beneficial potential in normal skin is still uncertain. A number of experimental studies indicate protective effects of beta-carotene against acute and chronic manifestations of skin photodamage. However, most clinical studies have failed to convincingly demonstrate its beneficial effects so far. Nevertheless, intake of oral beta-carotene supplements before sun exposure has been recommended on a population-wide basis. Recent studies on skin cells in culture have revealed that beta-carotene acts not only as an antioxidant but also has unexpected prooxidant properties. At present, there is an ongoing debate on the protective or potentially harmful role of beta-carotene in human skin. © 2001 Academic Press

Key Words: UV; carotenoids; beta-carotene; skin; oxidative stress; antioxidant; prooxidant.

Beta-carotene has repeatedly been called a “sun protectant” and been credited with preventing solar damage to skin. Consequently, intake of oral supplements in times of increased sun exposure has been suggested to be beneficial (1–3) and is now very popular among sun seekers. The essential function of carotenoids protecting cells against photosensitized reactions was first

hypothesized in the 1950s (4). Later, beta-carotene was found to prevent endogenous (chlorophyll) and exogenous photosensitization in bacteria, algae, and higher plants (5). Moreover, beta-carotene protected mice treated with hematoporphyrin (6) and humans suffering from photosensitivity to visible light (7). High-dose oral administration of beta-carotene has become a useful tool for therapy in patients with erythropoietic protoporphyria (EPP)² (8). This has led to the suggestion that beta-carotene might also have protective properties in normal skin and thus prevent solar damage.

Overexposure to sunlight provokes acute sunburn reaction which clinically manifests itself as erythema. Chronic exposure to sun leads to premature skin aging (“photoaging”) and increases the risk of both cutaneous melanoma and nonmelanoma skin cancer (NMSC) (9). Solar radiation has a strong oxidative component, and photooxidative stress has been directly linked to the onset of skin photodamage, as extensively reviewed by Fuchs (10). UVB radiation (280–320 nm) mainly damages DNA directly, due to an overlap with the absorption spectrum (11), and thus comprises a strong mutagenic potential. At the same time, it also has an oxidative component (12, 13). UVA radiation (320–400 nm), which contributes to up to 95% of total UV exposure (14), is not absorbed by DNA but it is a strong oxidant and considered the most important source of oxidative stress in human skin (15, 16).

The proposed beneficial effects of beta-carotene in skin have been mainly attributed to its antioxidant properties (2, 17–21). Experimental studies repeatedly found protection against UV-induced photodamage

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² Abbreviations used: EPP, erythropoietic protoporphyria; NMSC, nonmelanoma skin cancer; MED, minimal erythral dose; HO1, heme oxygenase-1; DMBA, *N*-Benzyldime thylamin; TPA, Phorbol-12-myristat-13-acetate.

such as erythema, immunosuppression, or skin cancer (22–26), though some authors found no beneficial effects (23, 27–30). So far, no clinical studies have been able to confirm these promising experimental results clearly (10). Clinical trials demonstrated protective effects of beta-carotene against acute skin reactions (2, 17, 31–33), but they failed to show any prevention of chronic photodamage (34–36). At present, both clinical and experimental data are highly inconsistent and some recent results also indicate the existence of potentially harmful effects of beta-carotene in UV-irradiated skin (37, 38). This review will briefly discuss the role of beta-carotene in human skin and its possible potential in protecting against the deleterious effects of solar radiation.

BETA-CAROTENE IN HUMAN SKIN

Carotenoids are part of the coloring system in human skin (39). Basal levels of beta-carotene in skin are usually rather low and were detected at ~0.03 to 0.4 nmol/g in wet tissue (1, 40) or at ~1.5 nmol/g when subcutaneous fat was included in samples (41). After consumption of a diet rich in carotenoids or oral supplementation, skin levels of beta-carotene are likely to increase, up to 17-fold over basal levels (1), which clinically manifests as yellowish complexion ("carotenoder-mia") (17, 31, 42–44).

In skin beta-carotene is mainly located in the epidermis (45, 46) where UVB radiation largely is absorbed (47). Beta-carotene does not absorb in the UVB range of light, and the oxidative component of UVB light is only weak. In dermal areas, where less beta-carotene is located, its antioxidant activity might be more important as they are the major target of UVA-induced oxidative stress. Near-ultraviolet light even reaches the cutaneous capillary system, indicating a possible field of action for beta-carotene in this compartment.

Exposure to sunlight reduces levels of beta-carotene in skin (1, 48). However, a single UV treatment did not significantly change concentrations of beta-carotene in skin but lycopene levels were decreased (49, 50). Overall, reduction of levels of beta-carotene and other carotenoids in skin might lower protection against UV damage. Consequently it may be hypothesized that increasing of beta-carotene concentrations before exposure to sunlight might provide a surplus and thus should reduce the risk of photodamage. Hata *et al.* (45) reported a correlation between carotenoid levels in skin and skin cancer. They found significantly lower carotenoid concentrations within perilesional, actinic keratosis and basal cell carcinoma sites when compared to skin from healthy subjects, and they suggested that reduced carotenoid levels in skin might predispose to the development of skin cancer.

Moderate carotenoder-mia is widely considered as beautiful and healthy complexion, particularly in infants, and the use of high doses of beta-carotene is considered relatively nontoxic (51). Nevertheless, it should be considered that a "safe range" of intracellular beta-carotene levels has not been determined yet, and it is not known whether beta-carotene accumulation in the skin might exert harmful side effects.

BETA-CAROTENE IN THE PREVENTION OF SKIN PHOTODAMAGE

The experience that beta-carotene inhibits photosensitized reactions in human skin (EPP) seemed to justify studies on the photoprotective effect of beta-carotene in normal skin. Initially, the results from clinical studies were rather disappointing. In 1972, Mathews-Roth and coworkers (33) published results of a clinical trial showing that long-term oral supplementation with beta-carotene (180 mg/day) increases the MED (minimal erythema dose) to a small but not significant extent. In other studies beta-carotene did not significantly protect against UVA, UVB, and PUVA erythema after single (120 mg/day) or chronic ingestion of beta-carotene (90–180 mg/day), although values of carotenoids in the plasma reached levels that had been shown to be protective in patients with EPP and although levels in skin increased accordingly and even manifested carotenoder-mia (49, 52).

A few clinical trials found significant preventive effects of beta-carotene against acute photodamage. For example, we reported reduced erythema formation in subjects which had been supplemented with beta-carotene (30 mg/day) for 10 weeks before and also during exposure to sunlight (2). In this study we also observed that beta-carotene significantly increased the density of Langerhans cells prior to sun exposure and prevented their decrease after irradiation. Long-term supplementation with beta-carotene (30 mg/day) also protected from UVA-induced immunosuppression, as determined by delayed-type hypersensitivity tests which are accepted assays to evaluate the individual sensitivity to UV light (2, 32, 53). Stahl *et al.* (17) reported significantly reduced erythema formation following carotenoid supplementation with mainly beta-carotene (25 mg/day) over 12 weeks. Additional supplementation with 335 mg of vitamin E per day increased the protective effect of beta-carotene, but not to a significant extent. Recently, Lee *et al.* (31) published data from individuals who had been supplemented over 24 weeks with increasing doses of carotenoids (30–90 mg/day) consisting of mainly beta-carotene. In these subjects, the MED rose with carotenoid intake to a small but significant extent. In contrast to our results a dose of only 30 mg/day carotenoids, however, did not significantly alter the MED. Besides, serum lipid peroxida-

tion, determined with a lipid peroxidation activity assay, was significantly diminished during carotenoid supplementation.

The effect of beta-carotene on erythema prevention should be considered rather modest. Besides, it might be questioned whether reduction or even complete suppression of erythema formation as a physiological response to overexposure to sunlight is indeed beneficial or desirable.

Regarding chronic manifestations of skin photodamage, there is a lack of data on the prevention of photoaging by beta-carotene. A few data on skin cancer prevention have been published, nevertheless, the results from observational studies are inconsistent (54–62), and data from randomized, controlled trials are scarce (34, 63). The Physicians' Health Study and its follow-up analysis showed that beta-carotene supplementation (50 mg/day) over a period of 12 years has no effect on the development of a first NMSC (34) and other malignant neoplasms (36). This corresponds to results from the Nambour Skin Cancer Prevention Trial, which showed that beta-carotene supplementation (30 mg/day) over 4–5 years does not alter incidence of a first NMSC, neither with nor without sunscreen use (35). Results from a few observational studies showed an association between increased beta-carotene levels in plasma and a reduced risk of a first NMSC (54, 56), but most studies found no such effect (55, 57–59, 64). Obviously it is at present not possible to determine whether there is a relationship between plasma beta-carotene levels and risk of a first NMSC.

Concerning secondary prevention of skin cancer, Greenberg *et al.* (63) conducted the only randomized controlled clinical trial on the effect of beta-carotene supplementation on NMSC development. Patients who had recently had NMSC were given beta-carotene (50 mg/day) over a period of 5 years. Though plasma levels of beta-carotene rose 10-fold, no protection against the development of a new skin cancer was found.

It may be concluded that the clinical data available do not clearly show any preventive effectiveness of beta-carotene supplementation on skin cancer.

BETA-CAROTENE FUNCTION IN SKIN EXPOSED TO SUNLIGHT

At present, the basic molecular and pathophysiological aspects of the interaction of beta-carotene, skin, and UV light are poorly understood. The absorption spectra of carotenoids typically occur in the near ultraviolet and visible light region of 360–550 nm (65); however, Sayre and Black (66) reported that even in yellowish skin not enough beta-carotene was present to filter hazardous radiation to a significant extent.

Furthermore, prevention of direct DNA damage by beta-carotene has been regarded less likely (66). What

seems more feasible is that beta-carotene acts as an antioxidant in the skin. Beta-carotene might provide protection against photosensitized reactions by quenching triplet sensitizers and singlet oxygen by energy transfer. Furthermore, beta-carotene might react with ROS such as oxygen radicals, peroxy radicals, and singlet oxygen (67–70). A variety of experimental studies investigated the antioxidant function of beta-carotene in the skin *in vivo* and *in vitro*. In rodents, beta-carotene was found to reduce lipid peroxidation (20, 71, 72), and topical application of beta-carotene reduced *in vivo* chemiluminescence (73, 74). It has also been demonstrated that beta-carotene quenches singlet oxygen-mediated photochemical reactions in rodent skin (75–77).

In cultured skin cells, a few *in vitro* studies have investigated the antioxidant potential of beta-carotene. Beta-carotene decreased photoinactivation of the enzymes catalase and superoxide dismutase, as well as protein cross linking (78). Furthermore, beta-carotene protected against membrane damage and lipid peroxidation (21). In rat kidney fibroblasts beta-carotene diminished UVA-induced catalase deactivation and lipid peroxidation (19), and in embryonic lung fibroblasts beta-carotene protected from UVA-induced cell damage (18). In this study, positive synergy effects were observed when beta-carotene treatment was combined with vitamin E or vitamins E plus C. Interestingly, treatment with vitamin E or C alone had no protective effects, and in cells exposed to UVB light, the protective effect of beta-carotene was minor.

It should be taken into consideration that beta-carotene might also act through one of its metabolites. Beta-carotene is likely to be degraded by photochemically generated ROS ("photobleaching") (79). Thus, the decrease observed in beta-carotene levels in skin and plasma might be due to photodegradation or photoisomerization. Whether such metabolites occur in the skin *in vivo* following exposure to solar radiation must be elucidated. Furthermore, as a provitamin A carotenoid, beta-carotene might be metabolized to retinoids via central cleavage by the enzyme 15,15'-dioxygenase, which has recently been found in mouse skin (80). Retinoic acid is considered the biologically most effective metabolite and it has been used successfully for prevention and treatment of skin photodamage (81–83). In human skin, expression of 15,15'-dioxygenase or formation of retinoic acid from beta-carotene has not been demonstrated yet.

Beside a variety of experimental data which it is claimed to explain beneficial or even preventive aspects of beta-carotene in skin, a few experimental data exist which document more or less detrimental effects. In mice, aggravating effects on skin cancer were found (84–86). For example, beta-carotene increased the formation of skin papillomas in mice (85) treated with

DMBA and TPA. Even so, beta-carotene inhibited the conversion of papillomas to carcinomas which indicates a chemopreventive effect of beta-carotene (85, 87). Black recently stated (84) that future studies using carotenoid supplementation should be carried out with caution until interactions of carotenoids and repair mechanisms of radicals are clarified.

We investigated the effect of beta-carotene on the cellular stress response in dermal fibroblasts on the level of gene expression. Using the UVA induction of heme oxygenase-1 (HO-1) as an accepted marker for oxidative stress (88), we studied the effect of beta-carotene (0.5 and 5.0 μM) on the HO-1 expression in irradiated cells. HO-1 induction is attenuated by cellular antioxidants. Accordingly, singlet oxygen quencher beta-carotene should diminish HO-1 induction in irradiated cells which is a consequence of photochemical generation of singlet oxygen (89). Unexpectedly, beta-carotene strongly enhanced the UVA induction of HO-1, which indicates that beta-carotene can have a prooxidative effect. In our study, the prooxidative effect of beta-carotene observed could be entirely suppressed by vitamin E (25 μM), but only moderately by vitamin C (100 μM) (37). As cosupplementation of the cells with vitamin E abolished the UV-induced increase of HO-1 in beta-carotene-treated cells, we assume that beta-carotene acts as a prooxidant and consequently causes membrane lipid peroxidation which can be prevented by vitamin E. It might be questioned whether this enhanced HO-1 expression could have beneficial effects, as HO-1 has been called an "emergency inducible defense pathway" for protection against UVA radiation in dermal fibroblasts (90). HO-1 is part of an adaptive response to UVA radiation which leads to protection against oxidative membrane damage (91) and mediates immunoprotection (92). According to present knowledge, induction of HO-1 is due to oxidative stress, e.g., UVA radiation, hydrogen peroxide, hypoxia, and hyperoxia. Our results show that beta-carotene may act as an amplifier of UVA-induced oxidative stress and subsequent increase of HO-1 expression.

Jones *et al.* (38) also published data demonstrating a prooxidative effect of beta-carotene in dermal fibroblasts which supports our findings. Beta-carotene (10 μM) was found to increase UVA/B-generated oxidative stress, resulting in increased release of superoxide anions and lipid peroxidation. Furthermore, beta-carotene reduced cellular adaptation to UV irradiation with a rise in catalase and superoxide dismutase activities and increase in cellular glutathione content.

CONCLUSIONS

Undoubtedly, beta-carotene is an important micronutrient with powerful biological effects. Nevertheless,

clinical studies as a whole have failed to persuade of an important role of beta-carotene as a photoprotector. The lack of an effect on skin cancer prevention in clinical trials might result from inadequate study protocols, as the duration of trials might have been too short and secondary prevention not the right target. However, even the results on acute manifestations of photodamage were ambiguous and mostly weak. In contrast to its effectiveness in the treatment of photosensitivity in patients with EPP, beta-carotene does not seem to be clinically beneficial as an oral sunscreen for healthy subjects. Beta-carotene might function as an antioxidant in human skin, but increasing levels in skin seems unlikely to modify the severity of skin photodamage. Experimental studies on the mechanism of action in skin cells *in vitro* have raised many questions and opened up a wide field of future research on the role of beta-carotene as a skin anti- or prooxidant.

It seems reasonable to assume that beta-carotene might combine both beneficial and detrimental effects in skin exposed to sunlight. The effects might depend on the biological endpoint investigated and the concentration of other antioxidants, e.g., vitamin E. In terms of using beta-carotene as a skin photoprotectant it should be pointed out that oral supplementation with beta-carotene as a single antioxidant might lead to an imbalance in the cutaneous antioxidant network and thus, as documented, exert possibly harmful effects on skin exposed to sunlight.

It seems questionable whether the present knowledge on beta-carotene action is sufficient to recommend intake of oral beta-carotene supplements or fortified food. Actually, it has been stated that there is at present no scientific evidence that high nutritional intake of beta-carotene from fruits and vegetables or a low-dose oral supplementation with beta-carotene might be harmful *in vivo* in general (93). From the data presented, we conclude that the use of single beta-carotene supplementation as an oral sun protectant should not be recommended at this time.

ACKNOWLEDGMENT

Our sincere apologies to those authors whose relevant publications are not cited due to space limitations.

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Protective Effects of Grape Seed Proanthocyanidins and Selected Antioxidants against TPA-Induced Hepatic and Brain Lipid Peroxidation and DNA Fragmentation, and Peritoneal Macrophage Activation in Mice

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ABSTRACT. 1. The comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) (25–100 mg/kg), vitamin C (100 mg/kg), vitamin E succinate (VES) (100 mg/kg) and β -carotene (50 mg/kg) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as production of reactive oxygen species by peritoneal macrophages, were assessed.

2. Treatment of mice with GSPE (100 mg/kg), vitamin C, VES and β -carotene decreased TPA-induced production of reactive oxygen species, as evidenced by decreases in the chemiluminescence response in peritoneal macrophages by approximately 70%, 18%, 47% and 16%, respectively, and cytochrome c reduction by approximately 65%, 15%, 37% and 19%, respectively, compared with controls.

3. GSPE, vitamin C, VES and β -carotene decreased TPA-induced DNA fragmentation by approximately 47%, 10%, 30% and 11%, respectively, in the hepatic tissues, and 50%, 14%, 31% and 11%, respectively, in the brain tissues, at the doses that were used. Similar results were observed with respect to lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates.

4. GSPE exhibited a dose-dependent inhibition of TPA-induced lipid peroxidation and DNA fragmentation in liver and brain, as well as a dose-dependent inhibition of TPA-induced reactive oxygen species production in peritoneal macrophages.

5. GSPE and other antioxidants provided significant protection against TPA-induced oxidative damage, with GSPE providing better protection than did other antioxidants at the doses that were employed. GEN PHARMAC 30;5:771–776, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Oxidative stress, lipid peroxidation, DNA fragmentation, grape seed proanthocyanidin extract, vitamin C, vitamin E succinate, β -carotene, zinc L-methionine, Swiss-Webster mice, 12-O-tetradecanoylphorbol-13-acetate (TPA)

INTRODUCTION

Free radicals have been implicated in more than 100 disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, tumor promotion and carcinogenesis, and AIDS (Ames, 1992; Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992; Kehrer, 1993; Pitot and Dragan, 1991). Free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis (Pitot and Dragan, 1991). Recent studies have demonstrated that environmental pollutants, radiation, pesticides, various medications, contaminated water and deep-fried and spicy foods, as well as physical stress, exhibit the ability to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation or procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in

gene expression and induction of abnormal proteins (Ames, 1992; Halliwell, 1996; Kehrer, 1993; Stohs and Bagchi, 1993).

Antioxidants/free-radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis and protect cells against oxidative damage (Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992). The consumption of edible plants, fruits and vegetables has been demonstrated to prevent the occurrence of a number of diseases in humans and animals (Hocman, 1989). Vegetables, fruits and their seeds are rich sources of vitamins C and E, β -carotene and protease inhibitors, compounds that might protect the organism against free radical-induced injury and diseases (Hocman, 1989).

Proanthocyanidins, naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark, are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics. Proanthocyanidins have been reported to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Afanas'ev *et al.*, 1989; Buening *et al.*, 1981; Kolodziej *et al.*, 1995). Furthermore, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation and capillary perme-

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ability and fragility and to modulate the activity of enzyme systems including cyclooxygenase and lipoxygenase (Bors and Saran, 1987; Kolodziej *et al.*, 1995). Proanthocyanidins are believed to be non-toxic. If they are absorbed and biologically active *in vivo*, they may prevent free radical-mediated cytotoxicity and lipid peroxidation and protect low-density lipoproteins from oxidation (Frankel *et al.*, 1993; Kinsella *et al.*, 1993).

A variety of proanthocyanidins have been shown to prevent the growth of breast cancer cells and to inhibit the enzymes taking part in the replication of rhino viruses (common cold) and HIV viruses (Hocman, 1989). The potential of isoflavones and lignans, also known as phytoestrogens, for preventing the development of hormone-dependent cancers such as breast and prostate cancer is attributed to their being weak estrogens (Hocman, 1989). Proanthocyanidins may exert these effects as antioxidants, potent free-radical scavengers and chelators of toxic heavy metals (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996).

In this study, we have assessed the comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) with vitamin C, vitamin E succinate (VES) and β -carotene *in vivo* against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA fragmentation in hepatic and brain tissues as well as against production of oxygen free radicals in peritoneal macrophages of mice.

MATERIALS AND METHODS

Chemicals

A commercially available dried, powdered GSPE (batch no. AV 609016) was obtained from InterHealth Nutritionals Inc. (Concord, CA). All other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

Animals and treatment

Female Swiss-Webster mice (20–25 g) were obtained from Sasco (Omaha, NE). The animals were housed in a controlled environment at 25°C with a 12-hr light and 12-hr dark cycle and were acclimated for at least 3–5 days before use. All animals were allowed free access to food (Purina Rodent Lab Chow No. 5001) and tap water. VES and β -carotene were dissolved in corn oil, whereas GSPE and vitamin C were dissolved in water. GSPE (25–100 mg/kg), vitamin C (100 mg/kg), VES (100 mg/kg) and β -carotene (50 mg/kg) were orally administered to groups of animals with the use of a feeding needle for 7 consecutive days. All treatments were conducted daily in the morning between 7:30 A.M. and 8:30 A.M. All groups of mice received an intraperitoneal (IP) injection of 1 ml of 3% thioglycolate (DIFCO Laboratories, Detroit, MI) broth 3 days before TPA treatment to elicit peritoneal macrophages (Witz and Czerniecki, 1989). TPA was administered on the 8th day 2 hr after the antioxidant treatment. Groups of mice were individually treated IP with 0.1 μ g TPA diluted in 1 ml of sterile phosphate-buffered saline (PBS) to induce an oxidative stress and were killed 2 hr posttreatment by cervical dislocation. Control animals received the PBS buffer. The peritoneal macrophage cells were isolated, the hepatic and brain tissues were quickly removed and the subcellular fractions were obtained as described elsewhere (Bagchi and Stohs, 1993). An approval (ARC no. 0313) from the Creighton University Animal Research Committee was obtained for this project.

Chemiluminescence assay

Chemiluminescence, as an index of reactive oxygen species production, was measured in a Chronolog Lumivette luminometer (Chronolog Corp., Philadelphia, PA).

The assay was conducted in 3-ml glass minivials. The vials were incubated at 37°C before measurement, and the background chemiluminescence of each vial was checked before use. Samples were preincubated at 37°C for 15 min, and 4 μ M luminol was added to enhance chemiluminescence. All additions to the vials as well as chemiluminescence-counting procedures were performed under dim lighting conditions. Results were presented as counts per unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30-sec intervals (Bagchi and Stohs, 1993).

Cytochrome *c* reduction assay

Superoxide anion production by peritoneal macrophages was measured by the cytochrome *c* reduction assay of Babior *et al.* (1973). The reaction mixtures contained 1 ml of macrophages (3×10^6 cells/ml) and 0.05 mM cytochrome *c*. The reaction mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures in ice. The mixtures were centrifuged at 1,500g for 10 min at 4°C, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 550 nm. Absorbance values were converted into nanomoles of cytochrome *c* reduced by using the extinction coefficient of 2.1×10^4 M/cm/15 min (Bagchi and Stohs, 1993).

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) associated with hepatic mitochondria and microsomes, as well as brain homogenates from control and treated animals were determined as an index of lipid peroxidation according to the method of Buege and Aust (1984) and as previously published by us (Bagchi and Stohs, 1993). Malondialdehyde was used as the standard. Absorbance values were measured at 535 nm, and an extinction coefficient of 1.56×10^5 M/cm was used.

DNA fragmentation

Liver and brain samples were homogenized in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000g for 20 min to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid, and 5.5 N perchloric acid was added to supernatant samples to reach a concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at 1,500g for 10 min to remove protein. Resulting supernatants were reacted with diphenylamine for 16–20 hr at room temperature, and absorbance was measured at 600 nm. DNA fragmentation is expressed as a percentage of total DNA appearing in the supernatant fractions. Treatment effects are reported as a percentage of control fragmentation (Ray *et al.*, 1993).

Statistical analysis

Data for each group were subjected to analysis of variance. Scheffe's S method was used as the *post hoc* test. The data are expressed as the mean \pm standard deviation of four animals. The level of statistical significance employed in all cases was $P < 0.05$.

RESULTS

Production of reactive oxygen species

TPA-induced *in vivo* production of oxygen free radicals in the peritoneal macrophages of mice was assessed by luminol-enhanced chemiluminescence and cytochrome *c* reduction assays. The results

TABLE 1. Production of reactive oxygen species by peritoneal macrophages based on chemiluminescence response and cytochrome c reduction after treatment of mice with TPA, and the comparative scavenging abilities of GSPE and selected antioxidants

Sample	Chemiluminescence (CPM/ 3×10^6 cells)	Cytochrome c reduction (nmoles/15 min/ 3×10^6 cells)
Control	995 \pm 156a	4.55 \pm 0.43a
Corn oil	937 \pm 88a	4.35 \pm 0.17a
Vitamin C (100 mg/kg)	1114 \pm 141a	5.27 \pm 0.50b
VES (100 mg/kg)	1771 \pm 139b	9.10 \pm 0.65c
Vitamin C + VES (100 mg/kg each)	1724 \pm 140b	9.02 \pm 0.58c
β -Carotene (50 mg/kg)	1198 \pm 118a	3.93 \pm 0.70a
GSPE (100 mg/kg)	1306 \pm 94a	5.07 \pm 0.58b
TPA	6031 \pm 591c	26.61 \pm 1.60d
TPA + vitamin C (100 mg/kg)	5081 \pm 335d	22.67 \pm 2.36d
TPA + VES (100 mg/kg)	3455 \pm 321e	18.03 \pm 0.83e
TPA + vitamin C + VES (100 mg/kg each)	2934 \pm 132e	14.24 \pm 1.52f
TPA + β -carotene (50 mg/kg)	5015 \pm 199d	21.81 \pm 1.38d
TPA + GSPE (25 mg/kg)	3592 \pm 211e	18.21 \pm 1.86e
TPA + GSPE (50 mg/kg)	2732 \pm 99e	12.65 \pm 1.41f
TPA + GSPE (100 mg/kg)	1724 \pm 268b	8.26 \pm 0.84c

Female Swiss-Webster mice were treated with a single dose of 0.1 μ g TPA after treatment with antioxidant(s) for 7 days. Peritoneal exudate cells (primarily macrophages) were analyzed for enhanced chemiluminescence and cytochrome c reduction. Each value represents the mean \pm SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

of the chemiluminescence and cytochrome c reduction assays for the production of reactive oxygen species by peritoneal exudate cells (primarily macrophages) are presented in Table 1.

The chemiluminescence produced by peritoneal macrophages from TPA-treated animals rapidly rises, reaching a maximum between 3 and 4 min, whereas macrophages from control animals reach a peak chemiluminescence at 3 min (data not shown). No significant increases in chemiluminescence were observed after treatment of the mice with either GSPE, vitamin C or β -carotene (Table 1). An approximately 1.8-fold increase in chemiluminescence was observed in the peritoneal macrophages of animals treated with VES alone. The succinate moiety has been previously shown to be responsible for this effect (Bagchi *et al.*, 1993).

A 6.1-fold increase in chemiluminescence was observed after treatment of the animals with TPA. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased the TPA-induced chemiluminescence in the peritoneal macrophages by 40%, 55% and 71%, respectively, compared with control values. Thus, a dose-dependent inhibition was demonstrated by GSPE. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased the TPA-induced chemiluminescence by 16%, 43%, 51% and 71%, respectively, compared with control samples. Administration of β -carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased the TPA-induced chemiluminescence response by 17% and 55%, respectively, relative to the control values.

The effect of TPA on the production of superoxide anion by peritoneal macrophages (determined by the cytochrome c reduction assay) also is presented in Table 1. The data are expressed as nanomoles of cytochrome c reduced 3×10^6 cells/15 min. GSPE, vitamin C and β -carotene had no effect on superoxide anion production in the absence of TPA. As previously noted with chemiluminescence, VES produced a significant increase (approximately 2.0-fold) in superoxide anion production (Table 1).

TPA administration increased the production of superoxide anion on the basis of cytochrome c reduction compared with the

cells from untreated animals by 5.9-fold (Table 1). GSPE induced a dose-dependent inhibition of the TPA-induced cytochrome c reduction. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased TPA-induced cytochrome c reduction by 32%, 53% and 69%, respectively, which still represented approximately 4.0-, 2.8- and 1.8-fold increases, respectively, above the control values. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced cytochrome c reduction by approximately 15%, 32%, 47% and 69%, respectively, compared with control samples. Administration of β -carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased TPA-induced cytochrome c reduction by approximately 18% and 48%, respectively, relative to the control values.

Lipid peroxidation

The effects of TPA and antioxidants on lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates are summarized in Table 2. No significant increases in lipid peroxidation were observed with GSPE, vitamin C or β -carotene. Approximately 1.4-, 1.2- and 1.2-fold increases in lipid peroxidation were observed in the hepatic mitochondria, hepatic microsomes, and brain homogenates, respectively, compared with control animals after treatment of the animals with VES, similar to previously reported observations (Bagchi *et al.*, 1993).

After treatment of mice with TPA, increases in lipid peroxidation of 2.7-, 2.9- and 3.1-fold were observed in hepatic mitochondria, hepatic microsomes and brain homogenates, respectively, compared with control values. Administration of 25, 50 and 100 mg GSPE/kg for 7 days to these animals decreased TPA-induced hepatic mitochondrial lipid peroxidation by 37%, 41% and 46%, respectively; in the hepatic microsomal fractions, decreases of 47%, 55% and 59% were observed, respectively, compared with control values. Approximately 46%, 53% and 61% decreases were demonstrated by GSPE

TABLE 2. TPA-induced lipid peroxidation in hepatic mitochondria and microsomes, and in brain homogenates of mice, and the comparative protective abilities of GSPE and selected antioxidants

	Lipid peroxidation (nmoles MDA/mg of protein)		
	Liver Mitochondria	Microsomes	Brain Whole homogenate
Control	2.17 ± 0.24a	2.76 ± 0.22a	1.62 ± 0.13a
Corn oil	2.30 ± 0.11a	2.59 ± 0.35a	1.69 ± 0.13a
Vitamin C (100 mg/kg)	2.38 ± 0.19a	2.85 ± 0.25a	1.68 ± 0.10a
Vitamin E succinate (VES) (100 mg/kg)	3.05 ± 0.14b	3.19 ± 0.29b	1.93 ± 0.17b
Vitamin C + (VES) (100 mg/kg each)	3.01 ± 0.17b	3.11 ± 0.15b	1.95 ± 0.10b
β-Carotene (50 mg/kg)	2.11 ± 0.12a	2.83 ± 0.11a	1.55 ± 0.12a
GSPE (100 mg/kg)	2.32 ± 0.14a	2.67 ± 0.32a	1.71 ± 0.22a
TPA	5.81 ± 0.34c	8.12 ± 0.84c	4.95 ± 0.32c
TPA + Vitamin C (100 mg/kg)	5.12 ± 0.34c	7.02 ± 0.42c	4.32 ± 0.23c
TPA + VES (100 mg/kg)	3.71 ± 0.39d	4.29 ± 0.44d	2.71 ± 0.49d
TPA + Vitamin C + VES (100 mg/kg each)	3.54 ± 0.49b,d	3.81 ± 0.42d,e	2.57 ± 0.35d
TPA + β-Carotene	5.41 ± 0.38c	7.17 ± 0.46c	4.54 ± 0.22c
TPA + GSPE (25 mg/kg)	3.68 ± 0.39d	4.33 ± 0.49d	2.69 ± 0.28d
TPA + GSPE (50 mg/kg)	3.43 ± 0.22d	3.65 ± 0.25e	2.33 ± 0.24d
TPA + GSPE (100 mg/kg)	3.13 ± 0.27b	3.31 ± 0.28b	1.94 ± 0.40b

Swiss-Webster mice were treated with a single dose TPA after receiving antioxidant(s) for 7 days. Thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation were determined on hepatic mitochondria and microsomes and on brain homogenates from control and treated animals. Malondialdehyde was used as the standard. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

against TPA-induced lipid peroxidation in the brain homogenates at these same concentrations.

Administration of vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C and VES (100 mg/kg each) and GSPE (100 mg/kg) for 7 days decreased TPA-induced hepatic mitochondrial lipid peroxidation by 12%, 36%, 39% and 46%, respectively, compared with control values, and 14%, 47%, 53% and 59% decreases, respectively, were observed in the hepatic microsomes. After treatment of the animals with these same antioxidants, 13%, 45%, 48% and 61% decreases, respectively, were observed against TPA-induced lipid peroxidation in brain homogenates. Administration of β-carotene (50 mg/kg) decreased TPA-induced hepatic mitochondrial and microsomal lipid peroxidation by approximately 7% and 12%, respectively; under these same conditions, an 8% decrease was observed in brain homogenate lipid peroxidation, compared with control values.

DNA fragmentation

Programmed cell death (apoptosis) has been identified as a selective process of physiological cell deletion. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei and extensive degradation of chromosomal DNA. Fragmentation of nuclear DNA is a biochemical hallmark of apoptosis (Schwartzman and Cidlowski, 1993).

TPA-induced DNA fragmentation in hepatic and brain tissues is summarized in Table 3, and the comparative protective abilities of various antioxidants are presented. TPA-induced 2.2- and 2.5-fold increases in DNA fragmentation in the hepatic and brain tissues of mice, respectively, compared with controls. No significant increases in DNA fragmentation were observed with GSPE, vitamin C or β-carotene. Approximately 1.3- and 1.4-fold increases in DNA fragmentation were observed in the liver and brain tissues, respectively,

compared with control animals after treatment of the animals with VES alone, similar to previous observations (Bagchi *et al.*, 1993).

A dose-dependent protective ability against TPA-induced DNA fragmentation was demonstrated by GSPE. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 days decreased TPA-induced hepatic DNA fragmentation by 36%, 42% and 47%, respectively, compared with control values, and DNA fragmentation decreased by approximately 32%, 44% and 50% in the brain tissues at these same concentrations. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced hepatic DNA fragmentation by 10%, 30%, 38% and 47%, respectively; under these same conditions, DNA fragmentation was reduced by 14%, 31%, 40% and 50% in brain tissues, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) for 7 days reduced TPA-induced hepatic and brain DNA fragmentation by 11%, relative to the respective control values.

DISCUSSION

Proanthocyanidins are a group of polyphenolic bioflavonoids ubiquitously found in fruits and vegetables. Proanthocyanidins have gained recent interest because of their broad pharmacological activity and therapeutic potential (Chen *et al.*, 1996; Hanefield and Herrmann, 1976; Masquelier *et al.*, 1979). Putative therapeutic effects of many traditional medicines may be ascribed to the presence of bioflavonoids (Brandi, 1992; Chen and Chan, 1996; Havsteen, 1983). The chemical properties of bioflavonoids in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996). For a proanthocyanidin or a bioflavonoid to be defined as an antioxidant, it must satisfy two basic conditions: (1) when present in low concentrations relative to the substrate to be oxidized, it can delay, retard, or pre-

TABLE 3. TPA-induced DNA fragmentation in the hepatic and brain tissues, and the comparative protective abilities of GSPE and selected antioxidants

Sample	Liver (%)	Brain (%)
Control	2.04 ± 0.30a	1.77 ± 0.28a
Corn oil	2.19 ± 0.31a	1.73 ± 0.22a
Vitamin C (100 mg/kg)	2.16 ± 0.34a	2.19 ± 0.31a,b
VES (100 mg/kg)	2.63 ± 0.24b	2.47 ± 0.36b
Vitamin C + VES (100 mg/kg each)	2.54 ± 0.36b	2.33 ± 0.31b
β-Carotene (50 mg/kg)	2.13 ± 0.22a	1.97 ± 0.26a
GSPE (100 mg/kg)	2.16 ± 0.31a	1.89 ± 0.31a
TPA	4.57 ± 0.51c	4.41 ± 0.28c
TPA + vitamin C (100 mg/kg)	4.12 ± 0.31c	3.80 ± 0.38d
TPA + VES (100 mg/kg)	3.18 ± 0.45d	3.03 ± 0.26e
TPA + vitamin C + VES (100 mg/kg each)	2.83 ± 0.23b,d	2.66 ± 0.21b
TPA + β-carotene (50 mg/kg)	4.06 ± 0.29c	3.92 ± 0.20d
TPA + GSPE (25 mg/kg)	2.94 ± 0.51b,d	3.00 ± 0.16c
TPA + GSPE (50 mg/kg)	2.67 ± 0.21b	2.49 ± 0.24b
TPA + GSPE (100 mg/kg)	2.43 ± 0.21b	2.22 ± 0.19b

Female Swiss-Webster mice were treated with a single dose of 0.1 µg TPA after receiving antioxidant(s) for 7 days. DNA fragmentation was measured spectrophotometrically by using Burton's reagent. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

vent autooxidation or free radical-mediated oxidative injury; and (2) the resulting product formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation (Shahidi and Wanasundara, 1992).

The biological, pharmacological and medicinal properties of the bioflavonoids and proanthocyanidins have been extensively reviewed (Jovanovic *et al.*, 1994; Rice-Evans *et al.*, 1996; Suzuki, 1993). Flavonoids and other plant phenolics are reported to possess, in addition to their free-radical scavenging and antioxidant activity, multiple biological activities including vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, anti-allergic, antiviral and estrogenic activities, as well as being inhibitors of the enzymes phospholipase A₂, cyclooxygenase and lipoxygenase (Rice-Evans *et al.*, 1996; Salah *et al.*, 1995).

The presence of various phenolic compounds, including phenoldienones, epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, *p*-coumaric acid, kaempferol, quercetin and myricetin, have been well established in proanthocyanidin extracts (Gonzalez *et al.*, 1982; Hanefield and Herrmann, 1976; Masquelier *et al.*, 1979; Rice-Evans *et al.*, 1996). It is well known that diets rich in fresh fruits and vegetables are protective against cardiovascular diseases and other oxidative stress-induced diseases and disorders including cancer (Chen and Chan, 1996; Halliwell, 1996; Halliwell *et al.*, 1992; Hocman, 1989). These chemoprotective properties have been attributed, in large part, to the presence of antioxidant nutrients vitamin C, vitamin E, β-carotene and mineral micronutrients. However, plant phenolics such as the bioflavonoids, proanthocyanidins and phenylpropanoids also may play a significant role. The proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants or by other mechanisms, contributing to anticarcinogenic or chemoprotective actions or both.

In this study, the protective abilities of GSPE, a commercially available grape seed proanthocyanidin extract, vitamin C; VES, a combination of vitamin C plus VES and β-carotene were assessed on TPA-induced oxidative tissue and DNA damage in the hepatic and brain tissues, as well as activation of peritoneal macrophages. The production of reactive oxygen species by peritoneal macrophages was assessed by measuring chemiluminescence and cyto-

chrome c reduction (Table 1). Cytochrome c reduction is a specific test for superoxide anion production (Ritchey *et al.*, 1981), whereas chemiluminescence is a general assay for the production of reactive oxygen species (Fisher and Adams, 1985). These assays clearly demonstrate the production of reactive oxygen species by peritoneal macrophages after administration of TPA and the comparative protective abilities of GSPE, vitamin C, a combination of vitamin C plus VES and β-carotene. GSPE demonstrated the best protection in the chemiluminescence assay compared with vitamin C, VES or β-carotene at the doses that were used. The combination of vitamin C and VES demonstrated better protection compared with the individual vitamins alone, which may be the result of regeneration of vitamin E from its oxidized form by vitamin C (Buettner, 1993). Similar results were obtained in the cytochrome c reduction assay (Table 1). These data indicate that GSPE as well as other antioxidants may be useful in preventing the *in vivo* production of reactive oxygen species.

Lipid peroxidation was assessed in the hepatic mitochondria and microsomes and in brain homogenate (Table 2); DNA fragmentation data for hepatic and brain tissues are presented in Table 3. Lipid peroxidation and DNA fragmentation serve as indicators of oxidative tissue damage. DNA fragmentation is believed to be a biochemical hallmark of apoptosis (programmed cell death), which plays a major role in developmental biology and in the maintenance of homeostasis in vertebrates (Schwartzman and Cidlowski, 1993). GSPE exhibited the best protection against TPA-induced hepatic mitochondrial and microsomal lipid peroxidation compared with the other antioxidants tested at the doses that were used (Table 2). A combination of vitamin C plus VES exerted better protection than did the corresponding individual vitamins. All antioxidants that were tested ameliorated TPA-induced increases in lipid peroxidation and DNA fragmentation in both brain and liver (Tables 2 and 3), with GSPE exhibiting the best protection compared with the other antioxidants.

These *in vivo* experiments demonstrate that GSPE is a better scavenger of free radicals and inhibitor of oxidative tissue damage than vitamin C, VES, a combination of vitamin C plus VES and β-carotene at the doses that were used. The results clearly demon-

strate that GSPE significantly attenuates TPA-induced oxidative stress in hepatic and brain tissues, as well as in peritoneal exudate cells (primarily macrophages). These data confirm that GSPE can significantly attenuate TPA-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as the enhanced production of oxygen free radicals in peritoneal macrophages. Furthermore, the results indicate that GSPE is bioavailable to vital target organs, including the liver and brain tissues and peritoneal exudate cells, and may therefore be useful in preventing the production of reactive oxygen species and oxidative tissue damage *in vivo*.

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